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The small molecule probe PT-Yellow labels the renal proximal tubules in zebrafish†

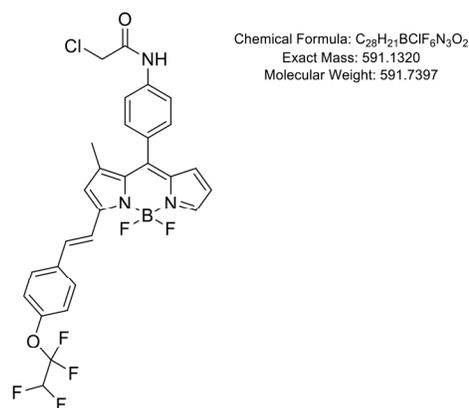**Veronika Sander,[‡a] Shantanu Patke,[‡a] Srikanta Sahu,[b] Chai Lean Teoh,[b] Zhenzhen Peng,[a] Young-Tae Chang,[b] Alan J. Davidson[*a]**

We report the development of a small fluorescent molecule, BDNCA3-D2, herein referred to as PT-Yellow. Soaking zebrafish embryos in PT-Yellow or intraperitoneal injection
10 **into adults results in non-toxic *in vivo* fluorescent labeling of the renal proximal tubules, the major site of blood filtrate reabsorption and a common target of injury in acute kidney injury. We demonstrate the applicability of this new compound as a rapid and simple readout for zebrafish kidney**
15 **filtration and proximal tubule reabsorption function.**

The kidney regulates fluid and electrolyte homeostasis, and waste excretion by blood filtration. The functional unit of the kidney, the nephron, consists of the glomerulus, where filtration occurs,
20 and a renal tubule that receives the ultrafiltrate. Due to the relatively non-selective nature of the blood filter, the majority of the ultrafiltrate is reabsorbed in order to reclaim useful molecules such as salt, glucose, and small proteins. Proximal tubule epithelial cells of the nephron perform the bulk of this recovery
25 and are also the target of damage by ischemic or toxic insults[1,2]. As such, there is a need for new research tools to study proximal tubule function and response to injury. The zebrafish represents an excellent vertebrate model organism for investigating mammalian renal disorders, due to the conservation in gene and
30 organ function and a plethora of genetic manipulation tools[3]. The zebrafish embryonic kidney (pronephros) consists of a pair of connected nephrons with a shared glomerulus and bilateral tubules. The tubules are divided into well-characterized segments including a highly absorptive proximal convoluted tubule (PCT)
35 segment that is separated from the glomerulus by a short neck region[4]. The PCT segment shows a high degree of ultrastructural and molecular conservation with the proximal tubule segments of mammals, including the presence of apical microvilli (brush border), which greatly expand the absorptive surface area of the
40 cells, and expression of cell surface proteins including the megalin/cubilin multi-ligand receptor complex[5]. The adult form of the zebrafish kidney, the mesonephros, develops during advanced larval stages by the addition of new nephrons to the distal segments of the pronephros. The fully formed mesonephros
45 consists of a highly-branched organization of nephrons with similar functional segmentation as the pronephros[6,7].
Central to studying renal function and injury in zebrafish is the labeling of the proximal tubule. At present, fluorescently-labeled

dextrans (10 – 40 kDa in size) are used to assay filtration and
50 tubular uptake. Molecules in this range are filtered by the glomerulus and endocytosed by the megalin/cubilin complex on the PCT segment where they accumulate in apically localized vesicles[5,8]. The drawback of using dextrans in this way is that they require injection into the embryonic circulation, which is
55 laborious and technically difficult. As a result, this technique is not readily applicable for large-scale forward genetic screening or when large numbers of animals need to be tested. Given this, we sought to identify an alternative reagent to assess the zebrafish kidney that is more amenable to high-throughput analysis.
60 Small fluorescent molecules have been widely used as imaging probes to label proteins or specific cells. In prior work, we developed highly diverse fluorescent compounds generated by the combinatorial modification of organic fluorescent scaffolds, a strategy which we dubbed diversity oriented fluorescence library
65 approach (DOFLA)[9]. As the design of DOFL is unbiased to any specific target, it can be evaluated with a broad range of molecules to maximize its chances of application in diverse fields. Indeed, extensive screening of DOFL against different systems have led to the discovery of 'hit' fluorescent compounds
70 for DNA, RNA, nucleotides, peptides, proteins and polysaccharides[10-15]. Previously, an image-based assay using DOFL molecules with a rosamine (RS) scaffold have been applied to zebrafish embryos and it was observed that many RS compounds label neurons located at the body surface, which
75 include the nose, internal ear and the neuromasts[16].

Chemical Formula: $C_{28}H_{21}BClF_6N_3O_2$
Exact Mass: 591.1320
Molecular Weight: 591.7397

Scheme 1 Chemical structure of PT-Yellow.

Here, we present a new compound identified by screening DOFL molecules, including some of the BDNCA series[17], for markers of kidney structures in zebrafish larvae. To screen the library, 3 days post-fertilization (dpf) zebrafish larvae were incubated in 100 nM
5 of the chemicals for 20 min, followed by wash-out and then imaging 24 hours later under epi-fluorescence. Compound BDNCA3-D2, which has a maximum absorbance at 548 nm and a fluorescence emission peak at 564 nm (yellow), was found to robustly label the proximal tubule (Fig. 1). We therefore named
10 this compound PT-Yellow, although its emission spectra extends into red wavelengths and can be detected with more commonly used red filter sets. The chemical structure of PT-Yellow is shown in Scheme 1 (ESI†).

To confirm the localization of PT-Yellow labeling, we made use
15 of the *Tg(PT::EGFP)*[18] and *Tg(nphs2::EGFP)*[19] transgenic lines that express enhanced green fluorescence protein (EGFP) in the proximal tubules and glomerulus, respectively. PT-Yellow labeling co-localizes with GFP+ proximal tubule cells (Fig. 1B-D) but was excluded from the glomerulus (Fig. 1F) and the neck
20 region (Fig. 1G, arrow). Higher magnification imaging of
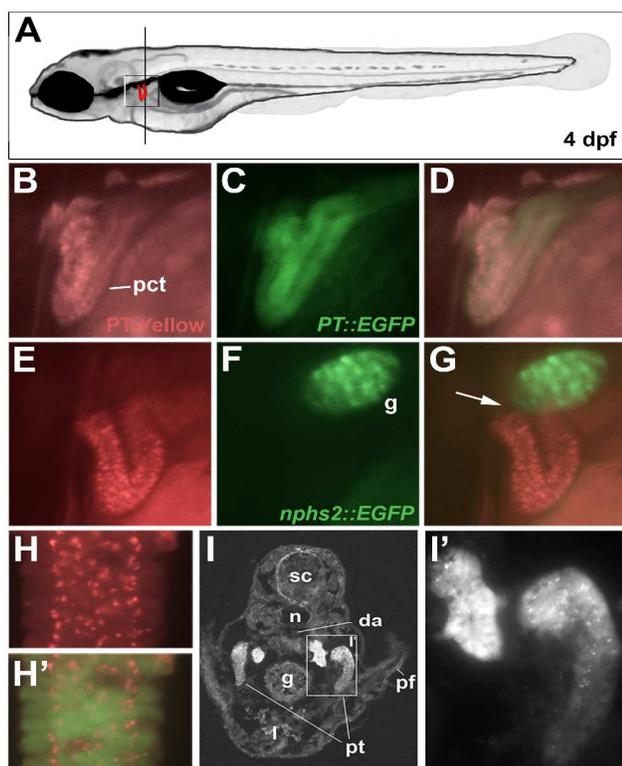
Figure 1 PT-Yellow labels the proximal tubule of larval zebrafish. (A) Schematic of a 4 dpf zebrafish larva. The area shown in B-G including the PCT segment of the pronephros (red), and the plane of section used for I and I' are highlighted. (B-D) PT-Yellow co-localizes with *PT::EGFP* expression in the PCT segment. (E-G) PT-Yellow is excluded from the glomerulus and neck (arrow). (H,H') PCT segment isolated from a PT-Yellow treated 4 dpf larva. Localization of PT-Yellow is visible at the apical side of the tubule. (I,I') Cross section of a PT-Yellow treated larva showing strong staining in the PCT. da, dorsal aorta; dpf, days post-fertilization; g, glomerulus; l, liver; n, notochord; pct, proximal convoluted tubule; pf, pectoral fin; pt, proximal tubule; sc, spinal cord.

dissected proximal tubules revealed that PT-Yellow displays an apically localized punctate staining pattern, consistent with being

taken up by endocytosis (Fig. 1H,H'). Possible mechanisms to account for PT-Yellow uptake include absorption into proximal
25 tubule cells by pinocytosis or via the megalin endocytic pathway, either directly or bound to carrier proteins. PT-Yellow labeling was stable following fixation, and analysis of cryosections revealed prominent labeling in PCT cells, as well as low level labeling in the liver (Fig. 1I,I').

30 PT-Yellow labeling at 100 nM did not affect survival, as treated and untreated larvae raised to 30 dpf showed similar survival rates (56% of DMSO-treated control fish, n=42/75; 63% of untreated fish, n=44/70; 64% of PT-Yellow-treated fish, n=48/75). Despite having no effect on mortality, we cannot rule-
35 out that PT-Yellow adversely affects cellular function such as flux through the endocytic pathway. In addition, no toxicity was observed when larvae were incubated continuously for 48 hrs in 100 nM PT-Yellow (data not shown). Significant toxicity was only observed when embryos were treated with PT-Yellow for 20
40 min at a concentration of 100 μM, manifesting as edema or death within 24 hrs in 22% of treated larvae (n=11/50), compared to 8.6% of DMSO-treated controls (n=3/35) and 5.8% of untreated controls (n=2/34). PT-Yellow labeling was durable; with proximal tubule fluorescence being readily detectable for at least
45 3 days following a single exposure (data not shown).

To determine the optimal timing of exposure to PT-Yellow and to examine how the compound was taken up into the animal, 5 dpf larvae were treated for 5, 10, 20, and 60 minutes, washed, and imaged, and then imaged again 24 hours later. Immediately after
50 the incubation for each of these periods (t=0), the compound was found to concentrate in the region of the developing gills, mouth and jaw (Fig. 2A-D). This distribution intensified with the length of treatment and also extended into the gut of the embryos treated for 60 minutes, suggesting that the compound was being taken up
55 via the gill vasculature and/or directly swallowed. In support of this, we observed PT-Yellow within the circulation of the 60 min treated animals (Fig. 2D, inset). At t=24, the compound was concentrated in the proximal tubules and in the gut for all incubation periods (Fig. 2A'-D'). While the 5 and 10 min periods
60 resulted in weak labeling of the kidney, the 20 min period gave robust proximal tubule staining (Fig. 2C'). There was no observable increase in labeling intensity in embryos incubated for 60 min (Fig. 2D'), indicating that a 20 min exposure time is sufficient to label the proximal tubules. Taken together, these
65 findings suggest that PT-Yellow is absorbed into the blood, filtered by the glomerulus, and taken up by the proximal tubules. Consistent with this, 3 dpf larvae co-treated with PT-Yellow and butanedione monoxime (BDM), which inhibits heart contractions and glomerular filtration, did not show PT-Yellow labeling of the
70 proximal tubules (Fig. 2E,F).

One of the advantages of the zebrafish model is the ability to perform large-scale forward genetic screens[20]. Such screens are generally dependent on rapid and reliable functional assays. To determine the suitability of PT-Yellow as a high-throughput assay
75 for assessing kidney and heart function, we examined embryos deficient in the *hnf1ba* and *hnf1bb* transcription factors, master regulators of kidney tubule differentiation and cardiac conduction between the atrium and ventricle[21,22]. *Hnf1ba/bb*-deficient embryos were achieved by injecting a morpholino oligomer
80 targeting *hnf1bb* into homozygous *hi1843* mutants, which harbors
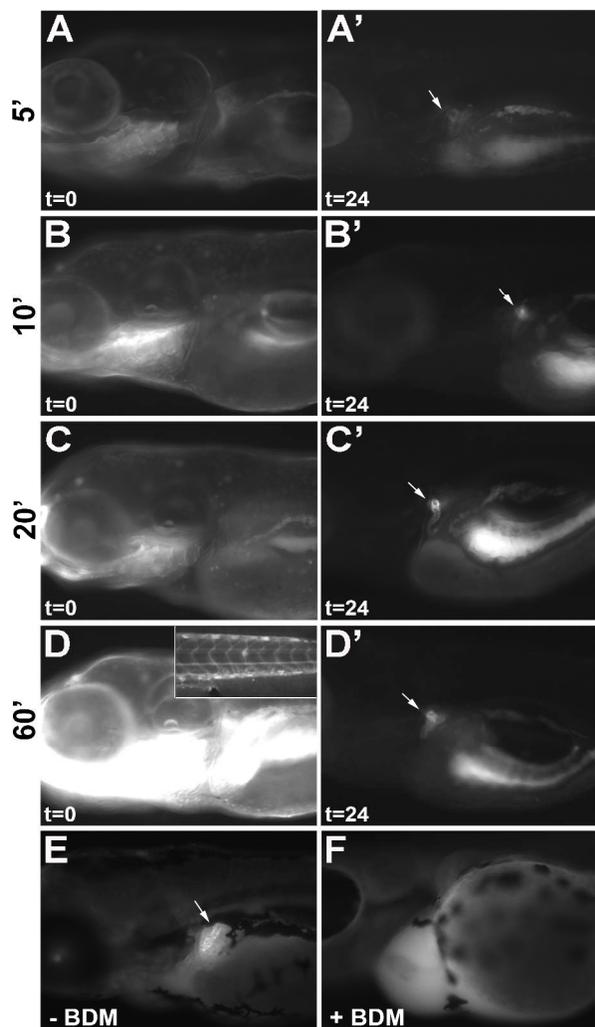
Figure 2 Time course of PT-Yellow uptake, distribution and filtration. (A-D) Uptake of PT-Yellow via the gills is visible immediately (t=0) upon treatment of 5 – 60 min. After 60 min, strong PT-Yellow staining is seen in the gut and circulation (inset in D). (A'-D') At t=24, weak labeling of the proximal tubules is detectable, even after only 5 min of PT-Yellow treatment (A', arrow). Strong PT-Yellow staining is achieved upon 20 min treatment (C'). (E,F) Elimination of heart beat and circulation pressure upon butanedione monoxime (BDM) treatment prevents glomerular filtration and PT-Yellow accumulation in the proximal tubule. A-D, 5 dpf; A'-D', 6 dpf; E and F, 4 dpf.

a recessive retrovirus-induced disruption of *hnf1ba*[23,24]. *Hnf1ba/bb*-deficient embryos develop pericardial edema by 3 dpf (Fig. 3), a relatively non-specific phenotype but one that is also associated with impaired circulation and blood filtration. PT-
5 Yellow treatment failed to label the proximal tubules in the mutants (Fig. 3D,D'), validating the use of PT-Yellow as a new tool to identify and screen for zebrafish mutants defective in kidney or heart function.

We further evaluated whether PT-Yellow could be used to label
10 proximal tubules of the adult zebrafish kidney (mesonephros). PT-Yellow was injected intraperitonally (IP) in *Tg(PT::EGFP)* transgenic fish and the kidney examined 24 hours later. Histological sections confirmed that PT-Yellow robustly labeled the mesonephric proximal tubules, as determined by co-
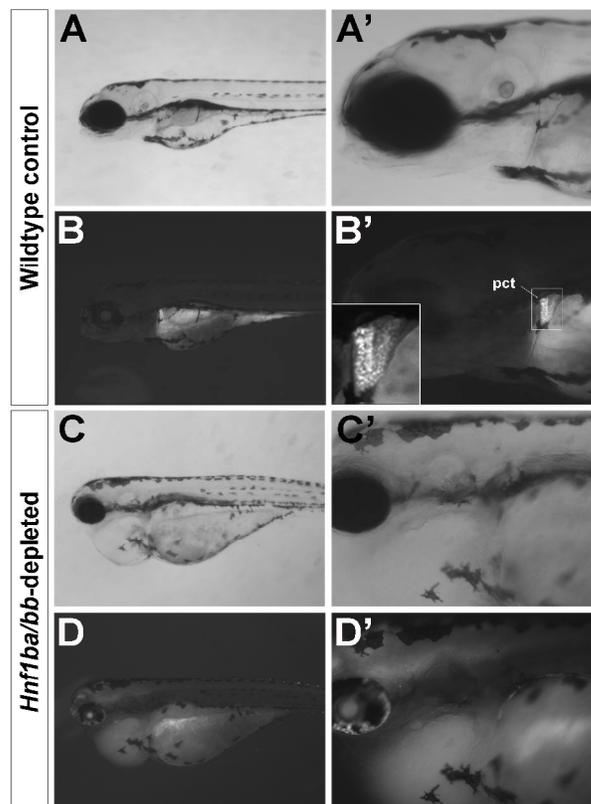
Figure 3 Detection of kidney and heart defects in zebrafish larvae. (A-B') Wildtype control larvae show PT-Yellow labeling in the PCT 24 hrs after treatment. (C-D') Zebrafish larvae lacking *hnf1ba* and *hnf1bb* develop cardiac edema at 4 dpf, presumably due to mis-conduction of the heart chambers and defective glomerular filtration. No PT-Yellow labeling is detectable in these mutants.

15 localization with EGFP. Similar to the larval kidney, PT-Yellow accumulated in large apically-localized vesicles, consistent with being taken up by endocytosis (Fig. 4A-C). We next investigated if PT-Yellow labeling could be used to monitor the level of proximal tubule cell damage induced by nephrotoxins. Prior
20 studies have shown that zebrafish proximal tubules can be ablated by IP-injection of gentamicin, followed by their recovery via a poorly understood regenerative mechanism[6,7]. PT-Yellow was IP-injected into *Tg(PT::EGFP)* adult zebrafish followed by an IP injection of gentamicin or water (control) 24 hours later.
25 Histological analysis at 6 days post injection (dpi) revealed a widespread loss of PT-Yellow-labeled/GFP+ proximal tubule cells in the gentamicin- but not control animals (Fig. 4). Some GFP+ cells that were not PT-Yellow labeled were also detected at this stage and likely correspond to newly regenerated proximal
30 tubule cells that have lost their initial labeling. Taken together, these results indicate that PT-Yellow has utility in adult kidney regeneration studies as a new tool to mark proximal tubule cells and to monitor their function.

In summary, we describe a newly developed chemical compound,
35 PT-Yellow, which is useful for *in vivo* labeling of proximal tubule cells in normal, mutant, and damaged zebrafish kidneys. Labeling of the proximal tubule is obtained upon simple short-term soaking of embryos in PT-Yellow-containing medium or via
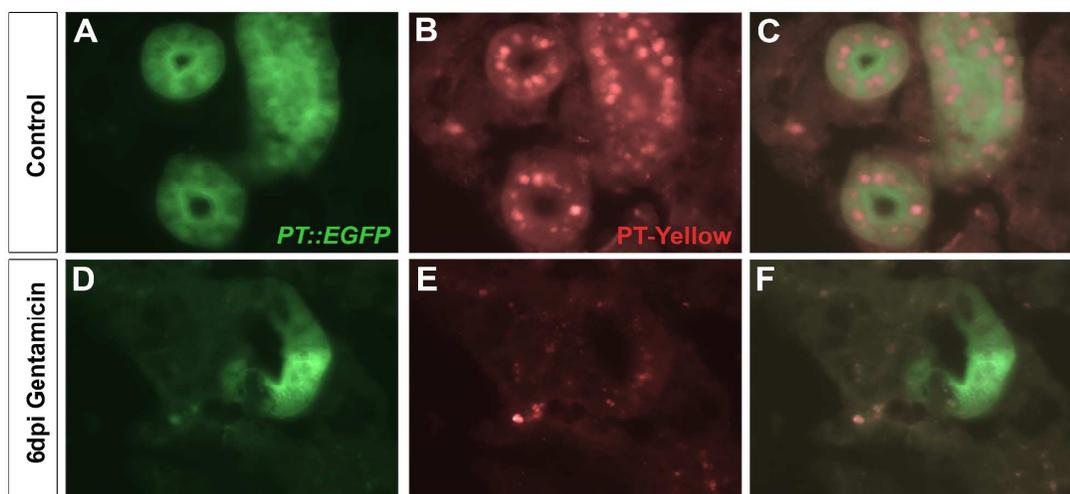
Figure 4 PT-Yellow labels proximal tubules in the adult zebrafish kidney. (A-C) PT-Yellow accumulates in vesicles located on the apical side of the proximal tubules (marked by *PT::EGFP*). (D-F) Gentamicin induced injury of proximal tubule cells results in loss of the PT-Yellow label, indicating functional damage of these cells. dpi, days post-injury.

IP injection into adult fish. The compound is non-toxic over a wide concentration range and labeling is preserved in fixed tissue sections. PT-Yellow provides zebrafish researchers with an additional reagent to label and test the function of proximal tubule cells and will be a valuable tool for future forward genetic screens.

Notes and references

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† Electronic supplementary information (ESI) available: Characterization of the new compound, additional graphs and detailed experimental procedures. See DOI: 10.1039/c000000x/
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